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## Translator's Declaration

I, Nicholas Hartmann, translator, having an office at 611 N. Broadway, Suite 509, Milwaukee, WI, 53202, declare that I am well acquainted with the English and German languages and that the appended document is a true and faithful translation of:

*Certified copy of German patent application no. 102 43 449.2, entitled*

*"Cars-Mikroskop und Verfahren zur Cars-Mikroskopie"*

*[CARS microscope and method for CARS microscopy]*

All statements made herein are to my own knowledge true, and all statements made on information and belief are believed to be true; and further, these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the document.

Date

*January 12, 2006*

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## CARS MICROSCOPE AND METHOD FOR CARS MICROSCOPY

The invention concerns a microscope for CARS microscopy, having means for generating a pump light beam and a Stokes light beam that can be directed coaxially through a  
5 microscope optical system onto a sample, and having a detector for detecting the detected light proceeding from the sample.

The invention furthermore concerns a method for CARS microscopy.

10 In scanning microscopy, a sample is illuminated with a light beam in order to observe the detected light emitted, as reflected or fluorescent light, from the sample. The focus of an illuminating light beam is moved in a specimen plane by means of a controllable beam deflection device, generally by tilting two mirrors, the deflection axes usually being  
15 perpendicular to one another so that one mirror deflects in the X direction and the other in the Y direction. Tilting of the mirrors is brought about, for example, by means of galvanometer positioning elements. The power level of the detected light coming from the specimen is measured as a function of the position of the scanning beam. The positioning elements are usually equipped with sensors to ascertain the present mirror position.

20 In confocal scanning microscopy specifically, a specimen is scanned in three dimensions with the focus of a light beam.

A confocal scanning microscope generally comprises a light source, a focusing optical system with which the light of the source is focused onto an aperture (called the "excitation  
25 pinhole"), a beam splitter, a beam deflection device for beam control, a microscope optical system, a detection pinhole, and the detectors for detecting the detected or fluorescent light. The illuminating light is coupled in via a beam splitter. The fluorescent or reflected light coming from the specimen travels back through the beam deflection device to the beam splitter, passes through it, and is then focused onto the detection pinhole behind which the  
30 detectors are located. Detected light that does not derive directly from the focus region takes a different light path and does not pass through the detection pinhole, so that a point datum is obtained which results, by sequential scanning of the specimen, in a three-dimensional image. A three-dimensional image is usually achieved by acquiring image data in layers, the path of the scanning light beam on or in the specimen ideally describing a meander  
35 (scanning one line in the X direction at a constant Y position, then stopping the X scan and slewing by Y displacement to the next line to be scanned, then scanning that line in the

negative X direction at constant Y position, etc.). To make possible acquisition of image data in layers, the sample stage or the objective is shifted after a layer is scanned, and the next layer to be scanned is thus brought into the focal plane of the objective.

- 5 Spectral detectors are often used for detection; these can be embodied, for example, as multi-band detectors as disclosed e.g. by German Unexamined Application DE 198 03 151.3 A1.

- 10 Coherent anti-Stokes Raman scattering (CARS) microscopy is a technique that is becoming increasingly important. One great advantage is that samples do not need to be labeled with dyes. In addition, living cells can be investigated.

- As compared to conventional Raman microscopy and known confocal Raman microscopy, in CARS microscopy a higher detected light yield can be obtained, troublesome secondary effects can be better suppressed, and detected light can be more easily separated from the illuminating light. Conventional confocal Raman spectroscopy requires a detection pinhole in order to achieve good axial resolution, as well as a high-resolution spectrometer. CARS, on the other hand, is a nonlinear optical process (four-wave mixing process). Similarly to the situation with multi-photon microscopy, in which two or more photons are absorbed simultaneously because the probability that multiple photons in correct phase will meet simultaneously is greatest at the focus due to the higher photon density, no detection pinhole is required. Without a detection pinhole, the same axial resolution as in multi-photon microscopy is achieved. Two lasers emitting light of different wavelengths ( $\nu_P$  and  $\nu_S$ , the pump laser and Stokes laser) are usually used for CARS spectroscopy;  $\nu_S$  should be tunable in order to generate a CARS spectrum  $\nu_{CARS}$  ( $\nu_{CARS} = 2\nu_P - \nu_S$ ,  $I_{CARS} \sim (I_P)^2 \cdot I_S$ ). FIG. 2 schematically depicts a term diagram of a CARS transition. If the frequency difference  $\nu_P - \nu_S$  matches the frequency difference between two molecular vibration states  $|1\rangle$  and  $|0\rangle$  in the sample, the CARS signal is then in fact amplified further. In microscope applications, the pump light beam and Stokes light beam are combined coaxially and focused together onto the same sample volume. The direction in which the anti-Stokes radiation is emitted is determined by the phase adaptation condition for the four-wave mixing process, as depicted schematically in FIG. 3.

- U.S. Pat. No. 4,405,237 "Coherent anti-Stokes Raman device" discloses an apparatus in which two pulsed laser beams, generated by two lasers and having different wavelengths in the visible region or the UV region of the spectrum, are used to illuminate a sample

simultaneously. With suitably selected wavelengths, the sample can be excited in such a way that it emits the characteristic coherent anti-Stokes Raman radiation.

U.S. Pat. No. 5,194,912 "Raman analysis apparatus" discloses a microscope that contains  
5 an adjustable interference filter in the detection beam path. The interference filter is adjustable in such a way that the portion of the detected light having the desired Raman lines arrives at the detector.

U.S. Pat. No. 6,108,081 "Nonlinear vibrational microscopy" discloses a method and an  
10 apparatus for microscopic CARS spectroscopy. This document discloses generation of a pump light beam with a titanium-sapphire laser, and a Stokes light beam with an optically parametric oscillator, which are combined into a coaxial illuminating light beam using a dichroic beam combiner. A regenerative amplifier is additionally provided in order to achieve sufficiently high pulse power levels.

15 Distinct disadvantages of the existing technology result from the limitations of the excitation light source, which usually is very complex because it comprises a pulsed laser (usually a titanium-sapphire laser), additionally a regenerative amplifier that makes low repetition rates (in the kHz range) unavoidable, and furthermore a complex and expensive optically  
20 parametric oscillator (OPO) that is pumped by a sub-beam of the pulsed laser. The known illumination arrangements are complex, expensive, and difficult to align; in particular, coaxial superimposition of the pump light beam and Stokes light beam requires a special alignment effort in order to ensure that the two beams are focused onto the same sample volume. The beam splitter used in the existing art for beam combination is usable only for specific  
25 combinations of pump light beam wavelength and Stokes light beam wavelength, representing a further disadvantage. If different wavelength combinations are selected, the beam splitter must be replaced—a long and laborious task.

A further disadvantage of the known apparatuses is the small available wavelength region,  
30 which also limits the range of samples that can be investigated.

It is therefore the object of the invention to describe a microscope for CARS microscopy which eliminates the problem of coaxial beam combination, and with which a very wide variety of samples can be flexibly investigated.

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This object is achieved by a microscope which is characterized in that the means for generating the pump light beam and the Stokes light beam encompass a laser and a microstructured optical element that spectrally broadens the light of the laser.

- 5 A further object of the invention is to describe a method for CARS microscopy with which a very wide variety of samples can be flexibly investigated.

The object is achieved by a method characterized by the following steps:

- 10 - generating a pump light beam and a Stokes light beam extending coaxially with the pump light beam, using means for generating a pump light beam and a Stokes light beam, the means for generating the pump light beam and the Stokes light beam encompassing a laser and a microstructured optical element that spectrally broadens the light of the laser;
- 15 - directing the pump light beam and the Stokes light beam onto a sample;
- detecting the detected light proceeding from the sample, using a detector.

The invention has the advantage that because they emerge from the same microstructured optical element, the pump light beam and the Stokes light beam always extend coaxially and  
20 complex beam combination is unnecessary.

When the wavelengths of the CARS excitation radiation are in the vicinity of the one-photon absorption of the molecules being investigated, the CARS emission is greatly exaggerated by resonance effects. Information about a specific molecule is obtained using the resonance  
25 exaggeration. It is therefore extremely important for the wavelengths of the pump light beam and the Stokes light beam to be matched accurately to the appropriate molecular transitions; this can be done easily and flexibly with the microscope according to the present invention.

The microstructured optical element offers the advantage that with suitable illumination, for  
30 example using the light of a pulsed laser that can be embodied as a Ti:sapphire laser, it emits light over a large wavelength region (approx. 400 - 1300 nm) with short pulses (fs or ps) and a high repetition rate. Because CARS is a nonlinear effect in which the instantaneous photon density should be as high as possible, short pulses are advantageous. A high repetition rate is preferably advantageous for rapid acquisition of images. The pump  
35 light beam and the Stokes light beam are extracted from the light that emerges from the microstructured optical element using a preferably variable filter. As a result of the

simultaneous generation of the light pulses, the latter emerge from the microstructured optical element exactly simultaneously and in phase. Means for adjusting the phase of the pump light beam and/or Stokes light beam are preferably provided. Compensation for shifts due to dispersion effects can be achieved, for example, using a delay section made of  
5 suitable optical material, or a dispersion compensation system.

In a preferred embodiment, means for selection of the pump light beam and means for selection of the Stokes light beam out of the spectrally broadened light are provided. The means for selection preferably contain an acoustooptical component that preferably is  
10 embodied as an acoustooptical tunable filter (AOTF). In a very particularly preferred embodiment, the means for selection directs the pump light beam and the Stokes light beam to the sample, and directs detected light proceeding from the sample to the detector. This configuration can preferably contain an apparatus as disclosed in German Unexamined Application DE 199 06 757 A1. An apparatus of this kind is very particularly advantageous  
15 because with it, any two wavelengths can be simultaneously cut out of the white-light spectrum. The wavelengths can also be varied separately by adjusting the HF frequency, and the undesired portions in the detected beam path can be effectively suppressed.

In a particular embodiment, the means for selection is adjustable in such a way that pump  
20 light beams and/or Stokes light beams of different wavelengths are selectable. The means for selection is preferably continuously adjustable.

In a preferred embodiment, at least one filter is provided that filters components having the wavelength of the pump light beam and the Stokes light beam out of the detected light  
25 proceeding from the sample, in particular in order to prevent those components from arriving at the detector and falsifying the measurement.

In a preferred embodiment of the scanning microscope, the microstructured optical element is constructed from a plurality of micro-optical structural elements that have at least two  
30 different optical densities. An embodiment in which the optical element contains a first region and a second region, the first region having a homogeneous structure and a microscopic structure of micro-optical structural elements being constituted in the second region, is very particularly preferred. It is additionally advantageous if the first region surrounds the second region.

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The micro-optical structural elements are preferably cannulas, webs, honeycombs, tubes, or cavities.

5 In another embodiment, the microstructured optical element comprises glass or plastic material and cavities arranged next to one another. Particularly preferred is the variant embodiment in which the microstructured optical element is made of photonic band gap material and is configured as a light-guiding fiber, an optical diode that suppresses the return reflection of the laser light beam attributable to the ends of the light-guiding fiber being provided between the laser and the light-guiding fiber.

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A very particularly preferred variant embodiment that is easy to implement contains as the microstructured optical element a conventional light-guiding fiber, having a fiber core diameter of approx. 9  $\mu\text{m}$ , that exhibits a taper along at least a portion. Light-guiding fibers of this kind are referred to as "tapered fibers." Preferably the light-guiding fiber is a total of 1 m  
15 long and exhibits a taper over a length of from 30 mm to 90 mm. In a preferred embodiment, the diameter of the light-guiding fiber in the tapered region is approx. 2  $\mu\text{m}$ . The fiber core diameter is correspondingly in the nanometer range.

20 In a very particularly preferred embodiment, the microscope is a scanning microscope, in particular a confocal scanning microscope. It preferably encompasses a scanning device that guides the pump light beam and the Stokes light beam over the sample. In a preferred embodiment, provision is made for the detected light to arrive at the detector via the scanning device, which can be embodied e.g. as an arrangement of galvanometer mirrors; in this embodiment, the microscope operates in descanned configuration. In another  
25 embodiment, the detected light is guided to the detector while bypassing the scanning device. In this embodiment, the microscope operates in non-descanned configuration. Since CARS is a four-wave mixing process, the detected light is usually emitted in the forward direction and accordingly is preferably detected using an additional downstream non-descanned detector.

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The detector preferably encompasses a multi-band detector or a spectrometer. One possible excitation is simultaneous irradiation at 600 nm and 800 nm. The CARS spectrum is then typically located at 480 nm. Using a multi-band detector, this spectrum can be separated without difficulty from a background fluorescence and from the excitation wavelengths.

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In a preferred embodiment of the method according to the present invention, the further step of selecting a pump light beam and/or a Stokes light beam out of the spectrally broadened light is provided.

- 5 In a particularly preferred embodiment, a resonance spectrum is acquired. From the resonance spectrum, it is possible to identify a wavelength combination of pump light beam and Stokes light beam at which a resonance maximum exists. If the sample contains several different substances, provision is preferably made that a resonance maximum is identified for at least two substances.

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The following procedure can be used to recover the CARS spectrum: in the first step,  $\lambda$  scans are acquired in descant configuration. Here  $\nu_P$  is first set to a transition, and  $\nu_S$  is tuned until the wavelength is found at which maximum resonance is identified ( $\nu_P - \nu_S$  then matches the frequency difference between two molecular vibration states  $|1\rangle$  and  $|0\rangle$  in the

- 15 sample). A suitable color filter or interference filter that is precisely matched to  $\nu_{\text{CARS}}$  is then selected (this being possible because all the relevant wavelengths are now accurately known), and three-dimensional image acquisition is begun in non-descant detection mode. In a further step, multiple molecules can be measured simultaneously, e.g. using a multi-band detector. A multi-channel CARS microscope is thereby obtained.

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This is possible only with a system of this kind, since every color component (even in the infrared) is present in the white light; with the acoustooptical component it is possible to switch back and forth rapidly between all possible wavelengths; and the multi-band detector allows  $\lambda$  scans to be performed with high resolution in order to determine the best

- 25 parameters. A mirror-coated specimen coverslip can preferably be used for acquisition of the  $\lambda$  scans. This is very particularly advantageous because the CARS signal is emitted from the sample substantially in the forward direction, i.e. in the direction of the illuminating light beam. The signal yield in the reverse direction can be considerably amplified in this fashion. The mirror coating can ideally comprise a cutoff filter that transmits the two excitation  
30 wavelengths and reflects the CARS wavelengths.

The subject matter of the invention is depicted schematically in the drawings and will be described below with reference to the Figures, identically functioning elements being labeled with the same reference characters. In the drawings:

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FIG. 1 shows a microscope for CARS microscopy;



FIG. 2 shows a term diagram of a CARS transition;

FIG. 3 illustrates the four-wavelength mixing process.

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FIG. 1 shows a microscope, configured as a confocal scanning microscope 1, that contains a laser 2 for generation of a light beam 5 of a first wavelength of 800 nm. The laser is embodied as a mode-coupled titanium-sapphire laser 3. Light beam 5 is focused by an incoupling optical system 7 into the end of a microstructured optical element 9 for wavelength modification, which is embodied as a light-guiding fiber made of photonic band gap material 11. An outcoupling optical system 13 is provided for collimation of broadened-wavelength light beam 15 emerging from the light-guiding fiber made of photonic band gap material 11. The spectrum of the modified-wavelength light beam is almost continuous over the wavelength region from 300 nm to 1600 nm, the light power level being largely constant over the entire spectrum; only in the region of the initial wavelength (800 nm) is a drastic power level exaggeration evident. Broadened-wavelength light beam 15 passes through a dielectric filter 17, constituting a means for suppression 16, which reduces the power level of the light component in the region of the first wavelength in broadened-wavelength light beam 15 to the level of the other wavelengths of the modified-wavelength light beam. The modified-wavelength light beam is then focused with optical system 19 onto an illumination pinhole 21 and subsequently arrives at a means 22 for selection which is embodied as an acoustooptical component 23 (AOBS) and functions as the main beam splitter. With means for selection 22, a pump light beam 24 and a Stokes light beam 26, each having a wavelength defined by the user, are selected. From means for selection 22, pump light beam 24 and Stokes light beam 26, which extend coaxially, travel to scanning mirror 25, which guides them through scanning optical system 27, tube optical system 29, and objective 31 and over sample 33. In descanned detection mode, detected light 35 proceeding from sample 33, which is depicted with dashed lines in the drawing, passes back through objective 31, tube optical system 29 and scanning optical system 27 to scanning mirror 25 and then to means for selection 22, passes through the latter, and after passing through detection pinhole 37 is detected with detector 39, which is embodied as a multi-band detector. For non-descanned detection, two further detectors 41, 43 are provided on the condenser side. Detected light 45 proceeding from the sample in the straight-ahead direction is collimated by condenser 47 and distributed by dichroic beam splitter 49 onto further detectors 41, 43 as a function of wavelength. Filters 51, 53 are provided in front of the

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detectors to suppress the components of the detected light that have the wavelengths of pump light beam 24 or Stokes light beam 26, or of other undesired fluorescent light.

5 The invention has been described with reference to a particular embodiment. It is self-evident, however, that changes and modifications can be made without thereby leaving the range of protection of the claims below.

Parts list

	1	Confocal scanning microscope
	2	Laser
5	3	Mode-coupled titanium-sapphire laser
	5	Light beam
	7	Incoupling optical system
	9	Microstructured optical element
	11	Photonic band gap material
10	13	Outcoupling optical system
	15	Broadened-wavelength light beam
	16	Means for suppression
	17	Filter
	19	Optical system
15	21	Illumination pinhole
	22	Means for selection
	23	Acoustooptical component
	24	Pump light beam
	25	Scanning mirror
20	26	Stokes light beam
	27	Scanning optical system
	29	Tube optical system
	31	Objective
	33	Sample
25	35	Detected light
	37	Detection pinhole
	39	Detector
	41	Further detector
	43	Further detector
30	45	Detected light
	47	Condenser
	49	Beam splitter
	51	Filter
	53	Filter

Claims

1. A microscope for CARS microscopy, having means for generating a pump light beam and a Stokes light beam that can be directed coaxially through a microscope optical system onto a sample, and having a detector for detecting the detected light proceeding from the sample,  
wherein the means for generating the pump light beam and the Stokes light beam encompass a laser and a microstructured optical element that spectrally broadens the light of the laser.
2. The microscope as defined in Claim 1, wherein the laser is a pulsed laser.
3. The microscope as defined in one of Claim 1 or 2, wherein means for selection of the pump light beam out of the spectrally broadened light are provided.
4. The microscope as defined in one of Claim 1 or 2, wherein means for selection of the Stokes light beam out of the spectrally broadened light are provided.
5. The microscope as defined in one of Claim 3 or 4, wherein the means for selection contain an acoustooptical component.
6. The microscope as defined in Claim 5, wherein the acoustooptical component is an AOTF.
7. The microscope as defined in one of Claims 3 through 6, wherein the means for selection directs the pump light beam and the Stokes light beam to the sample, and directs detected light proceeding from the sample to the detector.
8. The microscope as defined in one of Claims 1 through 7, wherein the means for selection is adjustable in such a way that pump light beams of different wavelengths are selectable.
9. The microscope as defined in one of Claims 1 through 8, wherein the means for selection is adjustable in such a way that Stokes light beams of different wavelengths are selectable.

10. The microscope as defined in one of Claim 8 or 9, wherein the means for selection is continuously adjustable.
- 5 11. The microscope as defined in one of Claims 1 through 10, wherein a filter is provided that filters components having the wavelength of the pump light beam and the Stokes light beam out of the detected light proceeding from the sample.
12. The microscope as defined in one of Claims 1 through 11, wherein means for adjusting the phase of the pump light beam and/or the Stokes light beam are provided.
- 10 13. The microscope as defined in one of Claims 1 through 12, wherein the microstructured optical element is constructed from a plurality of micro-optical structural elements that have at least two different optical densities.
- 15 14. The microscope as defined in one of Claims 1 through 13, wherein the microstructured optical element contains a first region and a second region, the first region having a homogeneous structure and a microstructure of micro-optical structural elements being constituted in the second region.
- 20 15. The microscope as defined in Claim 14, wherein the first region surrounds the second region.
16. The microscope as defined in one of Claims 1 through 15, wherein the microstructured optical element comprises glass or plastic material and cavities arranged next to one another.
- 25 17. The microscope as defined in one of Claims 14 through 16, wherein the micro-optical structural elements are cannulas, webs, honeycombs, tubes, or cavities.
- 30 18. The microscope as defined in one of Claims 1 through 17, wherein the microstructured optical element is made of photonic band gap material.
19. The microscope as defined in one of Claims 1 through 18, wherein the microstructured optical element is configured as a light-guiding fiber.
- 35 20. The microscope as defined in Claim 19, wherein the light-guiding fiber exhibits a taper.

21. The microscope as defined in one of Claims 1 through 20, wherein the microscope encompasses a scanning device.
- 5 22. The microscope as defined in Claim 21, wherein the scanning device encompasses a beam deflection device that guides the pump light beam and the Stokes light beam over the sample.
23. The microscope as defined in Claim 21, wherein the detector operates in descanned configuration.
- 10 24. The microscope as defined in Claim 21, wherein the detector operates in non-descanned configuration.
- 15 25. The microscope as defined in one of Claims 1 through 24, wherein the detector is a multi-band detector.
26. The microscope as defined in one of Claims 1 through 25, wherein the detector encompasses a spectrometer.
- 20 27. A method for CARS microscopy characterized by the following steps:
- generating a pump light beam and a Stokes light beam extending coaxially with the pump light beam using means for generating a pump light beam and a Stokes light beam, the means for generating the pump light beam and the Stokes light beam encompassing a laser and a microstructured optical element that spectrally broadens the light of the laser;
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- directing the pump light beam and the Stokes light beam onto a sample;
  - detecting the detected light proceeding from the sample, using a detector.
- 30 28. The method as defined in Claim 27, characterized by the further step of
- selecting a pump light beam out of the spectrally broadened light.
29. The method as defined in one of Claim 27 or 28, characterized by the further step of
- selecting a Stokes light beam out of the spectrally broadened light.

30. The method as defined in Claim 29, wherein the selecting encompasses a continuous varying of the wavelength of the Stokes light beam.
- 5 31. The method as defined in one of Claims 27 through 30, characterized by the further step of
- acquiring a resonance spectrum.
- 10 31. [sic] The method as defined in one of Claims 27 through 31, characterized by the further step of
- identifying the wavelength combination of pump light beam and Stokes light beam at which a resonance maximum exists.
- 15 32. The method as defined in Claim 31, wherein the sample contains several different substances; and a resonance maximum is identified for at least two substances.
33. The method as defined in Claim 32, wherein the detected light has several wavelengths that are simultaneously detected separately from one another.
- 20 34. The method as defined in one of Claims 27 through 33, wherein the microstructured optical element is constructed from a plurality of micro-optical structural elements that have at least two different optical densities.
- 25 35. The method as defined in one of Claims 27 through 34, wherein the microstructured optical element contains a first region and a second region, the first region having a homogeneous structure and a microstructure of micro-optical structural elements being constituted in the second region.
- 30 36. The method as defined in Claim 35, wherein the first region surrounds the second region.
37. The method as defined in one of Claims 27 through 36, wherein the microstructured optical element comprises glass or plastic material and cavities arranged next to one another.
- 35 38. The method as defined in one of Claims 27 through 37, wherein the micro-optical structural elements are cannulas, webs, honeycombs, tubes, or cavities.

39. The method as defined in one of Claims 27 through 38, wherein the microstructured optical element is made of photonic band gap material.
- 5 40. The method as defined in one of Claims 27 through 39, wherein the microstructured optical element is configured as a light-guiding fiber.
41. The method as defined in Claim 40, wherein the light-guiding fiber exhibits a taper.



Abstract

A microscope for CARS microscopy is disclosed, having means for generating a pump light beam and a Stokes light beam that can be directed coaxially through a microscope optical system onto a sample, and having a detector for detecting the detected light proceeding from the sample. The microscope is characterized in that the means for generating the pump light beam and the Stokes light beam contain a laser and encompass a microstructured optical element that spectrally broadens the light of the laser.

10 (FIG. 1)